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A Novel Production Method for High-Fructose Glucose Syrup from Sucrose-Containing Biomass by a Newly Isolated Strain of Osmotolerant *Meyerozyma guilliermondii*

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One osmotolerant strain from among 44 yeast isolates was selected based on its growth abilities in media containing high concentrations of sucrose. This selected strain, named SK-ENNY, was identified as *Meyerozyma guilliermondii* by sequencing the internal transcribed spacer regions and partial D1/D2 large-subunit domains of the 26S ribosomal RNA. SK-ENNY was utilized to produce high-fructose glucose syrup (HFGS) from sucrose-containing biomass. Conversion rates to HFGS from 310–610 g/l of pure sucrose and from 75–310 g/l of sugar beet molasses were 73.5–94.1% and 76.2–91.1%, respectively. In the syrups produced, fructose yields were 89.4–100% and 96.5–100% and glucose yields were 57.6–82.5% and 55.3–79.5% of the theoretical values for pure sucrose and molasses sugars, respectively. This is the first report of employing *M. guilliermondii* for production of HFGS from sucrose-containing biomass.

Keywords: *Meyerozyma guilliermondii*, fermentation, sucrose, sugar beet molasses, high-fructose glucose syrup

Introduction

High-fructose glucose syrup (HFGS) or high-fructose corn syrup (HFCS) is mainly produced by hydrolyzing the linked glucose molecules of starch to liberate free glucose monomers. To further increase the sweetness, a portion of the hydrolyzed glucose molecules are converted to fructose by using glucose isomerase, since fructose is the sweetest sugar found in nature [14]. HFCS is widely used in confectionaries and beverages, such as jams and cereal products. HFCS has steadily displaced the use of sucrose and glucose; the percentage of HFCS availability in the United States has increased from 16% in 1978 to 42% in 1998, and has remained consistent since then [21]. The major use of HFCS is in soft drinks, where 60% of the sucrose has been replaced by glucose and fructose [12].

Some microbes have the invertase enzyme, which converts sucrose to glucose and fructose, and ferment only the hydrolyzed glucose to ethanol, and consequently, fructose

remains without further metabolism. Thus, microbial glucose selection has been used as an alternative method for separating glucose from fructose during the production of fructose syrup from sucrose. Eighty percent of the theoretical maximum of fructose remained after a strain of *Tricholoma nudum* was cultivated in a medium containing sucrose [26].

Fructose syrup was also recovered from different concentrations of sugarcane syrup and molasses by a mutant of *Zymomonas mobilis* unable to utilize fructose [30]. Furthermore, elimination of glucose by conversion to ethanol from a synthetic glucose-fructose mixture and from hydrolyzed Jerusalem artichoke juice has been achieved using a mutant of *Saccharomyces cerevisiae* ATCC 36859, which cannot grow on fructose [10]. A mutant of *S. cerevisiae* ATCC 36858 lacking hexokinase activity to select for the utilization of glucose from hydrolyzed pure sucrose or molasses has also been intensively studied [1, 2].

Meyerozyma guilliermondii, formerly assigned as *Pichia*

guilliermondii, is the teleomorph state of *Candida guilliermondii* [20] and was reportedly a genetically heterogeneous complex belonging to the Saccharomycotina CTG clade [9]. *M. guilliermondii* is a flavinogenic yeast that is commonly known for the production of vitamin B2 (riboflavin) [31], and the genes affecting the regulation of riboflavin synthesis were recently identified [3]. Moreover, the properties and applications of osmotolerance were also studied by cultivating *M. guilliermondii* on the waste brine from kimchi production [5]. This species has recently gained attention for its potential in various biotechnological applications; for example, it has been found to enhance the production of volatile flavor compounds [32]. Furthermore, during the fermentation of soybean paste, *M. guilliermondii* produces isoflavone aglycones known to promote health by reducing the incidence of breast cancer and other common cancers, especially in Asian populations [16]. *C. guilliermondii* invertase enzyme was reported to have a potential for numerous industrial applications since the enzyme was active at high sucrose concentration with thermostability [24]. The availability of the complete genome sequence of *C. guilliermondii* strain ATCC 6260 [4] increases its usefulness for biotechnological studies and application, especially after the establishment of a convenient molecular toolbox including selectable markers and optimized transformation protocols [22].

In this study, we proposed a novel method for HFGS production from sucrose-containing biomass by direct fermentation, using a newly isolated strain of *M. guilliermondii*, named SK-ENNY. This strain may allow for the highly cost-effective production of HFGS by combining additional features, such as the assimilation of a wide range of carbon sources.

Materials and Methods

Strain Isolation, Cultivation, and Selection

A direct plating method was employed to isolate the yeast species using YPD medium (10 g/l of yeast extract, 20 g/l of peptone, 20 g/l of glucose) containing 20 g/l of agar. Filter-sterilized ampicillin (100 mg/l), chloramphenicol (100 mg/l), and cycloheximide (10 mg/l) were added as needed. Plates were incubated at 30°C for 2–4 days. Once a colony appeared, it was picked up and streaked onto a new plate of the same medium until a pure isolate was recovered. The cell/colony morphologies of the purified yeast isolates were investigated and monitored with a Nikon Eclipse E600 microscope. Yeast isolates were preserved on YPD slants at 4°C for short-term experimental use, and stocked in 20% glycerol at –80°C for long-term preservation.

Isolated species were screened for growth and fermentation

abilities of relatively high concentrations of sucrose. Only one species from among the 44 isolates showed sufficient osmotolerance, with the ability to grow on and ferment sugar beet molasses with a sucrose concentration of 200 g/l. This species was named SK-ENNY, and was subjected to further investigation.

DNA Extraction and Identification

SK-ENNY cells were suspended in a lysis buffer (2% Triton X-100, 1% sodium dodecyl sulfate, 100 mM NaCl, 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA) with glass beads and a mixture of phenol, chloroform, and isoamyl alcohol (25:24:1), and lysed by vortexing vigorously [13]. Liberated DNA was mixed with one-tenth volume of 3 M sodium acetate and precipitated with 2.5 volumes of ethanol, using ethachinmate (Nippon Gene, Toyama, Japan) as a carrier. Genomic DNA was purified using a QIAquick PCR purification kit (Qiagen, Tokyo, Japan) and used as a template to amplify a region of the ribosomal RNA gene using the ITS1 primer (5'-TCCGTAGGTGAACCTGCGG-3') as the forward primer, and the D1/D2-NL4 primer (5'-GGTCCGTGTTCAAGACGG-3') as the reverse primer with KOD Plus Neo polymerase (Toyobo, Osaka, Japan). PCR products were purified using the QIAquick PCR purification kit and subjected to sequencing. Identification was carried out according to White *et al.* [33] using ITS1 primer and ITS4 primer (5'-TCCTCCGCTTATTGATATGC-3') to sequence the conserved ITS1, 5.8S, and ITS2 regions by the 3031 genetic analyzer of Applied Biosystems (Tokyo, Japan) and ABI Prism BigDye Terminator v3.1 Cycle Sequencing kit. Sequencing data were submitted to NCBI/BLAST (<http://blast.ncbi.nlm.nih.gov/BLAST.cgi>) for sequence alignment, to construct a phylogenetic tree and to assign an accession number for the selected yeast isolate.

Physiological and Biochemical Characteristics

The ability for *M. guilliermondii* SK-ENNY to utilize glucose, fructose, galactose, mannose, sucrose, xylose, arabinose, xylitol, cellobiose, lactose, maltose, sorbitol, mannitol, rhamnose, raffinose, ethanol, methanol, inulin, and starch as a sole carbon source was measured by monitoring the optical density of cells grown on media containing 10 g/l of yeast extract, 20 g/l of peptone, and 20 g/l of the indicated carbon source for 48 h. Assimilation tests were also achieved by the replica plate method. Yeast strains were inoculated on a set of plates that contained different carbon or nitrogen sources in a basal agar medium, and the grown colonies were inspected and compared with negative control plates (without carbon or nitrogen sources) after incubation for 24–48 h [19]. Fermentation of these carbon sources was initiated following 24 h of cultivation on YPD medium. The ability to produce ethanol by fermenting each of these carbon sources was measured by high-performance liquid chromatography (HPLC) as described previously [15].

Physiological characters for identification of the selected strain was further carried out by checking the growth abilities on 50% and 60% glucose, and 10% NaCl/5% glucose. In addition, evaluation

of growth at 37°C, urea hydrolysis, starch formation, tolerance to 1% acetic acid, and acid production from glucose in Custer's chalk medium at 25°C after 2 weeks were carried out according to conventional identification methods of Kurtzman and Fell [18].

Fermentation and Optimization

M. guilliermondii SK-ENNY cultures were regularly cultivated on YPD medium until reaching an optical density at 600 nm (OD₆₀₀) of ~7, and then cells were harvested by centrifugation at 3,000 ×g to begin fermentation. To optimize for the initial cell biomass used for the fermentation process, three levels of cell density (OD₆₀₀=7.5, 10, and 12.5) were cultured in media containing 300 g/l of pure sucrose with 10 g/l yeast extract and 20 g/l peptone at 30°C with 150 rpm shaking. To optimize for temperature, cultures at the optimized initial biomass were cultured at various temperatures (25°C, 30°C, 37°C, and 42°C). Cell biomass was monitored along with progression of fermentation using a DEN-1B McFarland Densitometer (WAKEN Biotech, Kyoto, Japan). The sugar beet molasses used in this study was a kind gift from the BELKAS sugar factory (Dakahlia, Egypt). One aim of this study was to determine the maximum osmotic tolerance of the selected species to sucrose-containing biomass. The tolerance to the sucrose concentration of sugar beet molasses was used as a starting point for the determination of osmotolerance to pure sucrose, and to assess the behavior of *M. guilliermondii* SK-ENNY during the fermentation process. To accomplish this, fermentation was carried out on different concentrations of diluted sugar beet molasses (7.5%, 11.5%, 14.8%, 20%, 23%, and 30.5% (w/v)) without any additives, and different concentrations of sucrose (32%, 44%, 53%, and 61% (w/v)) containing 10 g/l of yeast extract and 20 g/l of peptone as a source of nitrogen, vitamins, amino acids, and other nutrients. Fermentation experiments were carried out in triplicate cultures with volumes of 100 ml in 300 ml Erlenmeyer flasks. Sucrose, glucose, fructose, and ethanol concentrations were determined by HPLC.

Results and Discussion

Strain Isolation, Selection, and Identification.

Many attempts have been made to produce sweetener syrups, such as HFCS and HFCS, using chemical and enzymatic methods [23]. Alternatively, employment of the microbes for selective elimination of glucose by conversion to ethanol from a hydrolyzed glucose-fructose mixture has been used to avoid chromatographic purification during the production of high-fructose syrups. Many species have been reported for their abilities to produce sweetener syrups, including *T. nudum* [26], mutant *Z. mobilis* [17, 30], and mutant *S. cerevisiae* [10]. Nonetheless, *M. guilliermondii*, which is ubiquitous [29] and the most common species found in raw honey [28], has never been reported or investigated for production of HFCS. The SK-ENNY strain

Table 1. Assimilation and fermentation of different carbon sources by *M. guilliermondii* SK-ENNY.

Carbon source	Assimilation	Fermentation
Glucose	+	+
Fructose	+	+
Galactose	+	+
Sucrose	+	+
Arabinose	+	-
Xylose	+	-
Cellobiose	+	-
Maltose	+	-
Mannose	+	-
Mannitol	+	-
Xylitol	+	-
Sorbitol	+	-
Raffinose	+	-
Lactose	-	-
Rhamnose	-	-
Ethanol	+	-
Methanol	-	-
Inulin	-	-
Starch	-	-

used in this study was selected from 44 yeast isolates obtained from different food products in Egypt. The selection was based on the osmotolerance to sugar beet molasses, which contained 200 g/l of sucrose (SBM200S) without any additives. The SK-ENNY strain isolated from mango juice can grow and ferment SBM200S. It showed a great ability to assimilate many different sugars as a carbon source (Table 1). It is also worthy to report that fructose and arabinose were the two carbon sources most efficiently utilized, as the growth rates on these sugars were almost double compared with other sugars. Furthermore, this strain efficiently fermented glucose, fructose, galactose, and sucrose to ethanol (Table 1). The physiological characters of *M. guilliermondii* SK-ENNY were identity with those reported [18], where the strain showed ability to grow well in 50% glucose but neither in 60% glucose nor in 10% NaCl/5% glucose. The strain also showed ability to grow at 37°C and produce acid from glucose. Moreover, it had no ability to hydrolyze urea, tolerate 1% of acetic acid, and form starch.

Genomic DNA of SK-ENNY was extracted and a region of the ribosomal RNA gene was amplified by PCR with ITS1 and NL4 primers. Sequences of the ITS1, 5.8S, and ITS2 regions and partial D1/D2 domains showed 99–100%

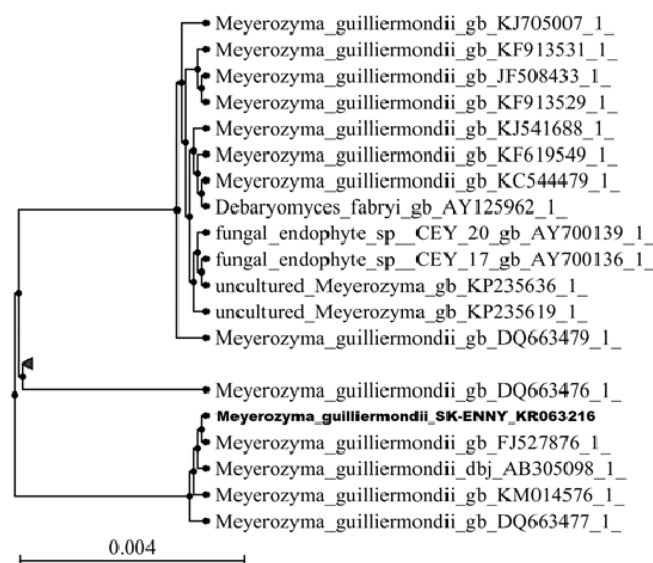


Fig. 1. Phylogenetic tree of isolated strain *M. guilliermondii* SK-ENNY using neighbor-joining analysis based on the ITS sequences.

The bar indicates the sequence dissimilarity value of 0.005 substitutions per site, visualized by the CLC sequence viewer.

identity with the sequence of the *M. guilliermondii* ribosomal RNA gene. A phylogenetic tree was constructed (Fig. 1), and the identification was confirmed and depicted using 0.05 maximum sequence differences of the neighbor-joining distance method [27] with the ITS sequence. The sequence of the *M. guilliermondii* SK-ENNY strain was submitted to the NCBI database and registered under the accession number KR063216.

Production of HFCS

During the selection of osmotolerant isolates and characterization of the properties of their fermentation at SBM200S, osmotolerant *M. guilliermondii* SK-ENNY strain was found to produce high fructose and glucose as a product of incomplete fermentation. Therefore, this strain was further investigated for production of HFCS by fermentation of sugar beet molasses at different initial sucrose concentrations, ranging from 75 to 305 g/l at the optimized temperature (30°C) (Fig. 2). Preliminary experiments revealed two distinctive behaviors during the fermentation process: (i) complete consumption of a relatively low initial concentration of sucrose, producing a maximum amount of HFCS before switching to fermentation processes that produced ethanol, and (ii) incomplete fermentation to ethanol at relatively high initial concentrations of sucrose, producing a relatively large amount of HFCS.

To examine the effects of organic compounds and minerals that may be present in sugar beet molasses and may act as inhibitors [6], media containing 10 g/l of yeast extract, 20 g/l of peptone, and 310–610 g/l of pure sucrose (Fig. 3) were used for fermentation. Compared with the previous experiments, higher concentrations of sucrose were used to determine the maximum osmolarity tolerance for pure sucrose, and also to confirm and compare the behavior of SK-ENNY grown on sucrose media or sugar beet molasses.

During fermentation of 50% (v/v) of crude molasses (SBM305S), which is equivalent 30.5% (w/v) of sucrose, SK-ENNY produced 25.7% (w/v) HFCS, representing a conversion of 85% of the total converted sucrose after 72 h (Fig. 2A). During this fermentation reaction, 77% of the sucrose in the molasses was converted after 48 h, and 88% after 72 h, reaching 91.1% after 96 h (Table 2 and Fig. 2A). The fructose concentration increased steadily over glucose, and the theoretical yields of fructose and glucose were 100% and 79.5%, respectively (Table 2). The maximum conversion rate and efficiency were observed at 48 h, where 90.0% of the consumed sucrose of the starting culture was converted to HFCS. The fructose ratio in this syrup represented 48.0% while glucose was 42.0%. The HFCS conversion rate was 2.68 g/l h⁻¹ under highly osmotic conditions, and the specific growth rate per hour reached 0.05 OD_{600nm} (Table 2). Ethanol was produced to a yield of 2.75% of consumed sucrose after 96 h. The conversion rates were tremendously higher (7.22 g/l h⁻¹) if 38% (v/v) crude sugar beet molasses, representing 23% (w/v) (SBM230S), was used as the starting material (Fig. 2B). Under these conditions, 94.2% of the sucrose in molasses was consumed, producing 18.8% HFCS (w/v) within 26 h. In this syrup, 49.1% of consumed sucrose of the starting material was converted to fructose, 35.9% to glucose, and 6.26% to ethanol (Table 2). The specific growth rate increased to 0.22 OD_{600nm} (Table 2). Continuous supplementation with crude molasses succeeded in increasing the HFCS ratio to 28.3% (w/v) within 80 h (data not shown). For fermentation of 33% (v/v) crude sugar beet molasses, representing 20% (w/v) (SBM200S), 95.4% of the sucrose in molasses was utilized within 20 h. The HFCS ratio reached 76.2% of converted sucrose within 20 h (Fig. 2C). In this syrup, fructose was obtained at 96.5% of the theoretical yield, while glucose was at 60.7%. Ethanol production represented 7.16% of the fermented sucrose (Table 2). The specific growth rate increased to 0.27 OD_{600nm} (Table 2). During fermentation with relatively low initial concentrations of sucrose (SBM150S, SBM115S, and SBM75S) the specific growth rate increased from 0.31 to 0.60 OD_{600nm}.

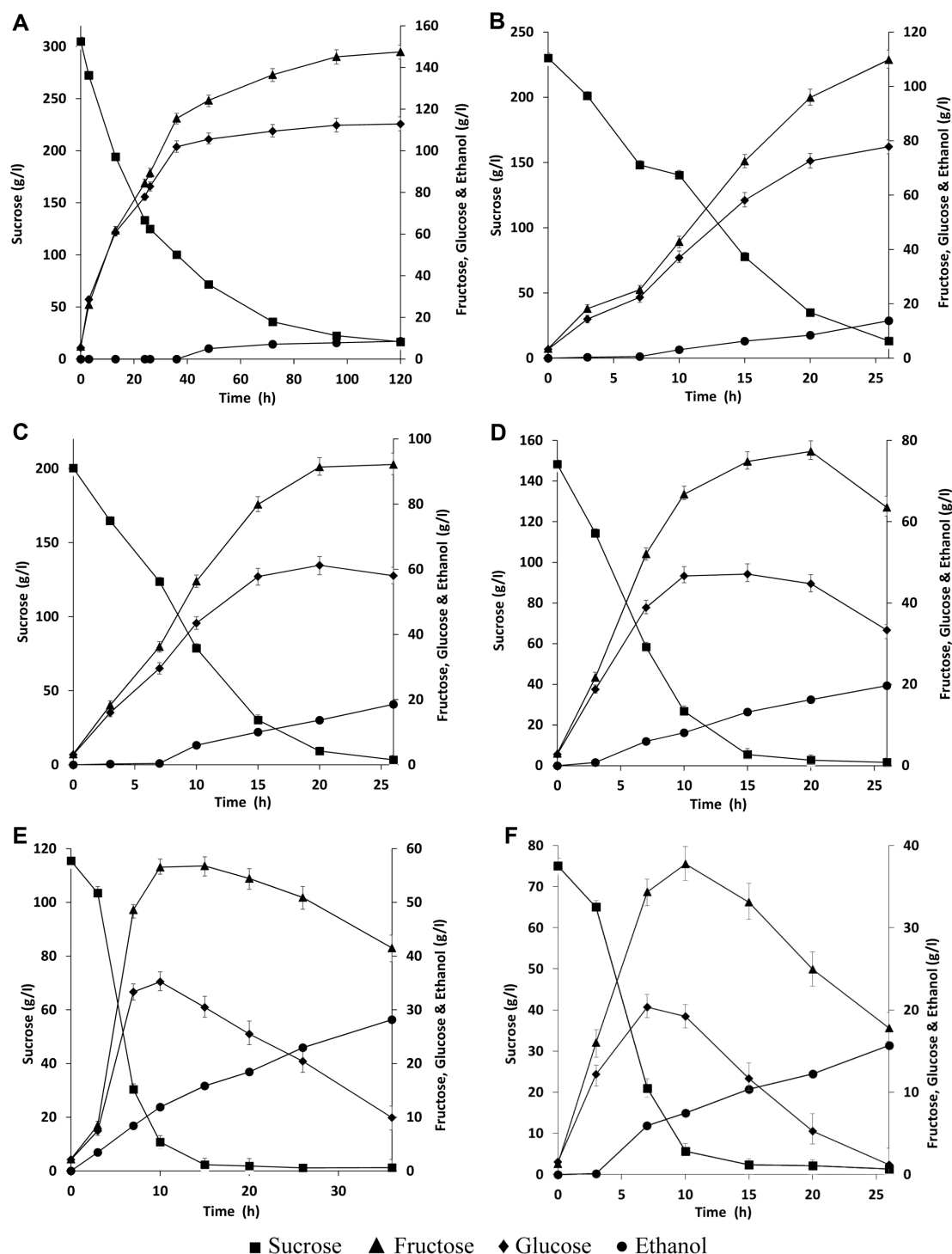


Fig. 2. Fermentation of sugar beet molasses and production of glucose, fructose, and ethanol using *M. guilliermondii* SK-ENNY with (A) SBM305S, (B) SBM230S, (C) SBM200S, (D) SBM150S, (E) SBM114S, and (F) SBM75S.

(Table 2). The ratio of ethanol was also higher, increasing from 11.2% to 21.5% of the fermented sucrose (Table 2). On the other hand, the yield of HFGS decreased together with

decreasing concentrations of sucrose. Furthermore, steady to complete fermentation of converted sugars to ethanol was observed after the maximum HFGS ratio was reached

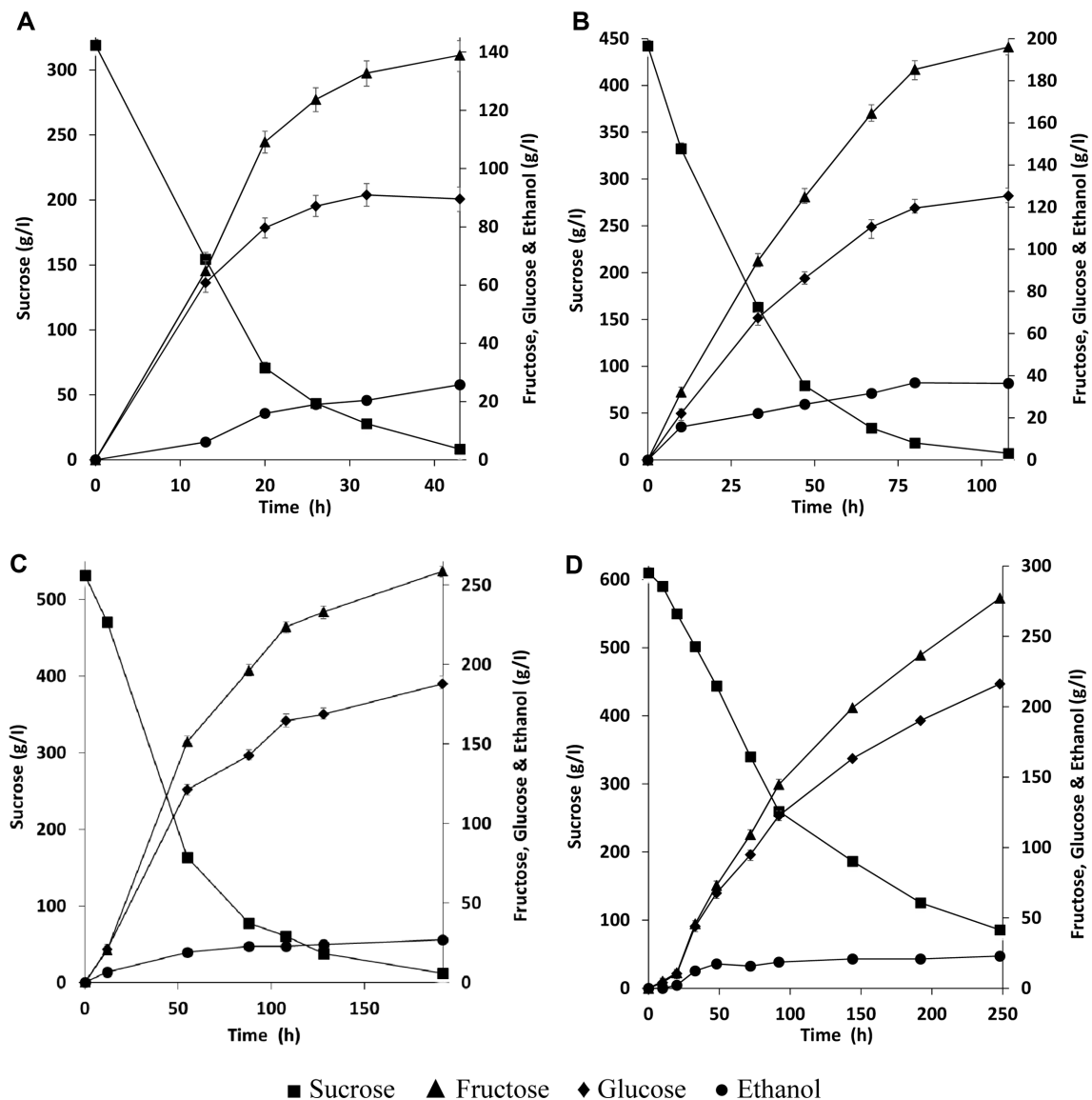


Fig. 3. Fermentation of sucrose, and production of glucose, fructose, and ethanol using *M. guilliermondii* SK-ENNY with (A) YP320S, (B) YP442S, (C) YP531S, and (D) YP610S.

(Figs. 2D–2F). Complete fermentation of 12.5%, 20.0%, and 25.0% (v/v) of crude sugar beet molasses, which represented 75 g/l, 115 g/l, and 148 g/l (w/v) of sucrose, respectively, to ethanol occurred within 72 h, yielding 0.46 g ethanol/g sucrose (data not shown).

To confirm and compare the behavior of *M. guilliermondii* under conditions of relatively high osmolality, the fermentation efficiencies and conversion rates were determined with pure sucrose-containing media. YP320S, YP442S, YP531S, and YP610S were used as media, and contained 32.0%, 44.2%, 53.1%, and 61.0% (w/v) pure

sucrose, respectively, with 10 g/l of yeast extract and 20 g/l of peptone. The *M. guilliermondii* SK-ENNY strain converted more than 97% of the sucrose in YP320S within 43 h, with an HFGS yield of 22.9% (w/v) representing 71.6% of the utilized pure sucrose (Fig. 3A). In this syrup, the fructose ratio was 44.7%, representing 89.4% of the theoretical yield, the glucose ratio was 28.8%, representing 57.6% of the theoretical yield, and 8.29% was ethanol. The specific growth rate reached 0.28 OD (Table 2). Optimization of the initial cell biomass for pure sucrose fermentation was required, and the best conditions resulted from using ~0.6–

Table 2. Sucrose conversion ratio, sucrose residual, theoretical fructose ratio, theoretical glucose ratio, HFGS conversion rate, HFGS ratio, ethanol ratio, fermentation time, initial cell biomass, biomass yield, and specific growth rate at different initial concentrations of crude sugar beet molasses and pure sucrose using *M. guilliermondii* SK-ENNY.

Type of sugar ^a	Initial sugars (g/l)	Sucrose conversion ratio (%)	Sucrose residual (g/l)	Theoretical fructose yield (%)	Theoretical glucose yield (%)	HFGS conversion rate (g/l h ⁻¹)	HFGS ratio (%) ^b	Ethanol ratio (%) ^c	Fermentation time (h)	Initial biomass (OD ₆₀₀)	Biomass yield (OD ₆₀₀)	Growth rate/h (OD ₆₀₀)
SBM	75.0	92.5	5.62	100	55.3	5.69	82.1	21.5	10	12.1	18.0	0.60
SBM	115	90.7	10.7	100	67.2	9.18	87.6	11.3	10	12.2	18.0	0.60
SBM	148	98.9	2.73	100	61.5	6.10	83.9	11.2	20	12.2	18.4	0.31
SBM	200	95.4	9.30	96.5	60.7	7.63	76.2	7.16	20	12.1	19.2	0.27
SBM	230	94.2	13.3	100	71.8	7.22	86.5	6.36	26	10.2	16.0	0.22
SBM	305	92.6	22.4	100	79.5	2.68	91.1	2.75	96	7.41	12.5	0.05
S	320	97.4	8.25	89.4	57.6	5.31	73.5	8.29	43	7.51	19.8	0.28
S	442	98.9	7.10	90.2	57.6	2.96	73.9	8.35	108	7.45	19.8	0.12
S	531	97.7	12.4	99.7	72.5	2.33	86.1	5.20	192	7.35	18.1	0.06
S	610	85.9	85.8	100	82.5	1.99	94.1	4.36	248	7.50	13.6	0.007

The data were calculated at the time indicated (fermentation time), which was the maximum HFGS ratio for each cultivation.

Data obtained from the mean of three independent experiments.

^aSBM: sugar beet molasses, S: sucrose supplemented by 10 g/l of yeast extract and 20 g/l of peptone.

^bHFGS ratio (%) = [HFGS produced (g/l) / Converted sucrose] × 100.

^cEthanol ratio (%) = [Ethanol produced (g/l) / Converted sucrose] × 100.

fold of the initial cell biomass used for sugar beet molasses fermentation (Table 2). For fermentation of YP442S (Fig. 3B), 98.9% of sucrose was converted to 32.1% (w/v) HFGS, representing 73.9% of total utilized sucrose, with an ethanol yield of 3.63% (w/v) at 108 h. Fructose and glucose ratios revealed 89.4% and 57.6% of the theoretical yields, respectively. The specific growth rate was retarded to 0.12 OD (Table 2). Fermentation of YP531S (Fig. 3C) showed that more than 97% of the sugar was converted to 44.7% (w/v) of HFGS, representing 86.1% of the initial sucrose after 192 h. Fructose and glucose ratios of this HFGS were 48.7% and 35.4%, respectively, while the ethanol yield was 5.20%. The conversion rates decreased with increasing concentrations of initial sugar provided, with conversion rates of 5.31, 2.96, 2.33, and 1.99 g/l h⁻¹ of YP320S, YP442S, YP531S, and YP610S, respectively. Although reductions were observed in the conversion rates and specific growth rates, especially at 53.1% and 61.0% (w/v) initial sucrose concentrations, the efficiency of fermentation increased to 86.1% and 94.1%, respectively, (Table 2; Figs. 3C and 3D). In YP610S, the fructose ratio reached 100%, while glucose was 82.5% of the theoretical limit of converted sucrose.

The above results strongly suggest that *M. guilliermondii* SK-ENNY strain possesses a highly expressing periplasmic invertase enzyme, which rapidly converts sucrose to glucose

and fructose. The conversion of sucrose to glucose and fructose at concentrations that ranged from 7.5% to 61% might be explained by the presence of invertase enzyme that is an analog of the glycosylated thermostable invertase enzyme of anamorph *C. guilliermondii*, which is active at high sucrose concentrations [24]. Moreover, production of HFGS by *M. guilliermondii* SK-ENNY can be explained by the action of higher utilization affinity to converted glucose than fructose; in fact, the strain performed a higher conversion of glucose to ethanol than fructose preceded by the highest conversion of sucrose during fermentation of equal concentrations (40 g/l) of sucrose, glucose, and fructose mixtures (data not shown).

Furthermore, the production of HFGS by *M. guilliermondii* SK-ENNY was dependent on the initial sucrose concentration, with productivity increasing at high initial sugar concentrations (Table 2). Osmotic tolerance might be a limiting factor for the productivity and stability of HFGS at higher sugar concentrations. By contrast, the instability and reduction of HFGS observed were likely due to the conversion of hydrolyzed sugars to ethanol at lower sugar concentrations. At high initial sugar concentrations, the inhibitory effects of having a high concentration of substrates were clearly observed for the fermentation of glucose and fructose to ethanol. These data are in agreement

with previous studies [7, 25], where the utilization and fermentation of fructose were greatly affected by high concentrations of glucose.

To the best of our knowledge, this is the first study to conclude that *M. guilliermondii* can be used to produce HFCS from sucrose-containing biomass, although many other strains, either native or genetically modified, were used to ferment glucose only during fermentation of sucrose to produce fructose and ethanol. This HFCS was stable at higher concentrations, probably due to the inhibitory effects of high concentrations of hydrolyzed fructose and glucose. With SBM305S and YP610S, the concentrations of sucrose that were the maxima for osmotic tolerance, the sucrose conversion ratios of the total initial sugars were 92.6% at 96 h and 85.9% at 248 h, respectively. Using SBM305S and YP610S, HFCS represented 91.1% and 94.1% of the total consumed sucrose, respectively, with fructose ratios of 100% for both, and glucose ratios of 79.5% and 82.5% of the theoretical yields, respectively. These efficiencies were comparable to those of the previous studies using other organisms. The sucrose hydrolysis efficiencies of *S. cerevisiae* ATCC 36858 were 98% in a 629 g/l sucrose-containing synthetic medium [1] and 100% in a 312 g/l sucrose-containing sugar beet molasses [2]. The efficiencies with *Z. mobilis* ranged between 73% and 94% in media containing between 350 and 462 g/l of sucrose [8, 11, 30]. *M. guilliermondii* SK-ENNY may have some advantages over these other strains. *M. guilliermondii* utilizes a wide range of carbon source, including most hexose and pentose sugars. There is also the possibility to improve the production rates and efficiencies by overexpressing the endogenous sucrase gene, and/or knocking out the alcohol dehydrogenase gene. Further refinements can be made in future studies, such as introducing an exogenous glucose isomerase gene, or simply adding external enzymes directly to the mixture to isomerize glucose. This strategy may result in an increased fructose ratio and be effective for further improvements of this method.

In summary, *M. guilliermondii* SK-ENNY was selected for its ability to produce HFCS from sucrose-containing biomass using a direct fermentation method. *M. guilliermondii* SK-ENNY was osmotolerant, and HFCS production rates increased with higher initial sugar concentrations. In the resulting syrups, the fructose ratio reached 100%, while the glucose ratio reached 82.5% of the theoretical maximum values for sugar conversion. This study is a starting point for using *M. guilliermondii* to produce HFCS; the SK-ENNY strain has great potential for industrial-scale applications, considering its relatively low production of ethanol and the

ability of the species to utilize a wide range of carbon sources.

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References

- Atiyeh H, Duvnjak Z. 2001. Production of fructose and ethanol from media with high sucrose concentration by a mutant of *Saccharomyces cerevisiae*. *J. Chem. Technol. Biotechnol.* **76**: 1017-1022.
- Atiyeh H, Duvnjak Z. 2002. Production of fructose and ethanol from sugar beet molasses using *Saccharomyces cerevisiae* ATCC 36858. *Biotechnol. Prog.* **18**: 234-239.
- Boretsky YR, Pynyaha YV, Boretsky VY, Fedorovych DV, Fayura LR, Protchenko O, *et al.* 2011. Identification of the genes affecting the regulation of riboflavin synthesis in the flavinogenic yeast *Pichia guilliermondii* using insertion mutagenesis. *FEMS Yeast Res.* **11**: 307-314.
- Butler G, Rasmussen MD, Lin MF, Santos MA, Sakthikumar S, Munro CA, *et al.* 2009. Evolution of pathogenicity and sexual reproduction in eight *Candida* genomes. *Nature* **459**: 657-662.
- Choi MH, Park YH. 1999. Growth of *Pichia guilliermondii* A9, an osmotolerant yeast, in waste brine generated from kimchi production. *Bioresour. Technol.* **83**: 251-253.
- Chotineerant S, Wansuksri R, Piyachomkwan K, Chatakanonda P, Weerathaworn P, Sriroth K. 2010. Effect of calcium ions on ethanol production from molasses by *Saccharomyces cerevisiae*. *Sugar Tech.* **12**: 120-124.
- D'Amore T, Russell I, Stewart GG. 1989. Sugar utilization by yeast during fermentation. *J. Ind. Microbiol.* **4**: 315-324.
- Doelle W, Greenfield PF. 1985. Fermentation pattern of *Zymomonas mobilis* at high sucrose concentrations. *Appl. Microbiol. Biotechnol.* **22**: 411-415.
- Dujon B. 2010. Yeast evolutionary genomics. *Nat. Rev. Genet.* **11**: 512-524.
- Duvnjak Z, Koren DW. 1987. Production of fructose syrup by selective removal of glucose from hydrolyzed Jerusalem artichoke juice. *Biotechnol. Lett.* **9**: 783-788.
- Edye LA, Johns MR, Ewings KN. 1989. Fructose production by *Zymomonas mobilis* in fed-batch culture with minimal sorbitol formation. *Appl. Microbiol. Biotechnol.* **31**: 129-133.
- Glinemann WH, Irausquin H, Park YK. 1986. Evaluation of health aspects of sugars contained in carbohydrate sweeteners. Report of sugars task force, 1986. *J. Nutr.* **116**: 1-216.
- Hanna M, Xiao W. 2000. Isolation of nucleic acids, pp. 15-

20. In Xiao W (eds.), *Yeast Protocols*, 2nd Ed. Vol. 313. Humana Press, New Jersey.
14. Hanover LM, White JS. 1993. Manufacturing, composition, and applications of fructose. *Am. J. Clin. Nutr.* **58**: 724-732.
15. Khattab SMR, Saimura M, Kodaki T. 2013. Boost in bioethanol production using recombinant *Saccharomyces cerevisiae* with mutated strictly NADPH-dependent xylose reductase and NADP⁺-dependent xylitol dehydrogenase. *J. Biotechnol.* **165**: 153-156.
16. Kim WC, So JH, Kim SI, Shin JH, Song KS, Yu CB, Kho YH, Rhee IK. 2009. Isolation, identification, and characterization of *Pichia guilliermondii* K123-1 and *Candida fermentati* SI, producing isoflavone β -glycosidase to hydrolyze isoflavone glycoside efficiently, from the Korean traditional soybean paste. *J. Appl. Biol. Chem.* **52**: 163-169.
17. Kirk LA, Doelle HW. 1994. Simultaneous fructose and ethanol production from sucrose using *Zymomonas mobilis* 2864 co-immobilized with invertase. *Biotechnol. Lett.* **16**: 533-538.
18. Kurtzman CP, Fell JW. 1998. Summary of species characteristics, pp. 627-634. In Kurtzman CP, Fell JW (eds.). *The Yeasts: A Taxonomic Study*, 4th Ed. Elsevier, Amsterdam.
19. Kurtzman CP, Fell JW, Boekhout T, Robert V. 2011. Methods for isolation, phenotypic characterization and maintenance of yeasts, pp. 87-110. In Kurtzman CP, Fell JW, Boekhout, T (eds.). *The Yeasts: A Taxonomic Study*, 5th Ed. Elsevier, Amsterdam.
20. Kurtzman CP, Suzuki M. 2010. Phylogenetic analysis of ascomycete yeasts that form coenzyme Q-9 and the proposal of the new genera *Babjeviella*, *Meyerozyma*, *Millerozyma*, *Priceomyces*, and *Scheffersomyces*. *Mycoscience* **51**: 2-14.
21. Marriott BP, Cole N, Lee E. 2009. National estimates of dietary fructose intake increased from 1977 to 2004 in the United States. *Nutrition* **139**: 1228-1235.
22. Papon N, Courdavault V, Clastre M. 2014. Biotechnological potential of the fungal CTG clade species in the synthetic biology era. *Trends Biotechnol.* **32**: 167-168.
23. Parker K, Sale M, Nwosu V. 2010. High fructose corn syrup: production uses and public health concerns. *Biotechnol. Mol. Biol. Rev.* **5**: 71-78.
24. Plascencia-Espinosaa MB, Santiago-Hernández A, Pavón-Orozco P, Vallejo-Becerra V, Trejo-Estrada S, Sosa-Peñadoc A, et al. 2014. Effect of deglycosylation on the properties of thermophilic invertase purified from the yeast *Candida guilliermondii* MpIIIa. *Process Biochem.* **49**: 1480-1487.
25. Reed MC, Lieb A, Nijhout FH. 2010. The biological significance of substrate inhibition: a mechanism with diverse functions. *Bioessays* **32**: 422-429.
26. Reusser F, Gorin PAJ, Spenser JFT. 1960. The production of fructose as a residue of sucrose fermentation by *Tricholoma nudum*. *Can. J. Microbiol.* **6**: 17-20.
27. Saitou N, Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**: 406-425.
28. Saksinchai S, Suzuki M, Chantawannakul P, Ohkuma M, Lumyong S. 2012. A novel ascosporogenous yeast species, *Zygosaccharomyces siamensis*, and the sugar tolerant yeasts associated with raw honey collected in Thailand. *Fungal Divers.* **52**: 123-139.
29. Spencer JFT, Spencer DM. 1997. Ecology: where yeasts live, pp. 33-58. In Spencer JFT, Spencer DM (eds.). *Yeasts in Natural and Artificial Habitats*. Springer, Berlin.
30. Suntinanalert P, Pemberton JP, Doelle HW. 1986. The production of ethanol plus fructose sweetener using fructose utilization negative mutant of *Zymomonas mobilis*. *Biotechnol. Lett.* **8**: 351-356.
31. Tanner F, Vojnovich C, Lane JM. 1945. Riboflavin production by *Candida* species. *Science* **101**: 180-185.
32. Wah TT, Walaisri S, Assavanig A, Niamsiri N, Lertsiri S. 2013. Co-culturing of *Pichia guilliermondii* enhanced volatile flavor compound formation by *Zygosaccharomyces rouxii* in the model system of Thai soy sauce fermentation. *Int. J. Food Microbiol.* **160**: 282-289.
33. White TJ, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, pp. 315-322. In Innis MA, Gelfand DH, Sninsky JJ, Taylor TJ (eds.). *PCR Protocols: A Guide for Methods and Applications*. Academic Press, New York.